Biochimica et Biophysica Acta, 569 (1979) 259—266

BBA 68777

A STUDY OF THE REACTION CATALYSED BY ALGINATE LYASE VI FROM THE SEA MOLLUSC, *LITTORINA* sp.

V.V. FAVOROV, E.I. VOZHOVA, V.A. DENISENKO and L.A. ELYAKOVA

Pacific Institute of Bio-organic Chemistry, Far East Science Centre, U.S.S.R. Academy of Sciences, Vladivostok-22 (U.S.S.R.)

(Received January 2nd, 1979)

Key words: Alginate lyase; Polymannuronate, Uronic acid; Mannuronic acid

Summary

The molecular weight of polymeric alginic acid digested by alginate lyase (poly(1,4-β-D-mannuronide) lyase, EC 4.2.2.3) was determined at various stages of the lysis. Low molecular weight fragments were detected only after 60—100% lysis. Some high molecular weight fragments remained intact even after addition of a fresh aliquot of enzyme to the digest.

The enzyme showed maximal activity at pH 5.6 in 0.05 M salt. Enzyme activity was stimulated by addition of 7.5 mM $CaCl_2$ and 0.2 M NaCl, when the pH optimum was between 8 and 8.5.

Only mannuronic acid was detected at the reducing end of fragments after exhaustive enzymolysis, reduction and hydrolysis.

On studying the reaction products by NMR, a double-bond signal (σ = 5.98 ppm) was observed. A considerable decrease in intensity of the D-mannuronic acid residue signal was detected after hydrolysis of alginate lyase VI on poly-(ManUA-GulUA), but not poly(GulUA).

The results suggest that alginate lyase VI may be an endoalginate lyase that splits glycoside bonds only between two mannuronic acid residues.

Hydrolysis by polyuronide lyases of the glycosidic bond linking the two uronic acids generally found in natural heteropolyuronides and representing C-5 epimers (D-mannuronic- and L-guluronic acids in alginate, D-glucuronic and L-iduronic acids in chondroitin, hyaluronate and heparin) involves formation of a single 4-deoxy-5-ketouronic acid [1—5]. Alginic acid and alginate lyases are suitable substrate models for investigating the specificity of the enzymes.

Abbreviation: Gul, guluronic acid.

Alginic acid (which constitutes 1,4-bonded D-mannuronic and L-guluronic acids) may contain four types of linkage: ManUA-GulUA, GulUA-ManUA, GulUA-GulUA and ManUA-ManUA. An unambiguous determination of the type of linkage split by an enzyme would initially require determination of the monomers on the reducing and non-reducing ends of the resultant oligomers.

We established that purified alginate lyase VI (poly(1,4-β-D-mannuronide) lyase, EC 4.2.2.3) caused a rapid decrease of viscosity of the polymeric substrate and a slow increase of the reducing capacity in the reaction mixture; this is characteristic of enzymes with endo-type action. Alginate lyase VI was found to split poly(ManUA) and the alginate most rapidly; at the same time, it produced slower splitting of poly(ManUA-GulUA) and had no effect on poly-(GulUA).

The present work investigates the nature of the products after exhaustive hydrolysis of alginic acid and determines the nature of the cleaved bonds.

Materials and Methods

Enzyme isolation, estimation of enzymic activity, analytical methods and reagents were all as described previously [6].

Gel filtration of alginate lyase VI end products. The reaction mixture (4 mg/ml sodium alginate, 0.2 M NaCl, 0.2% NaN_3 and enzyme solution) in 0.05 M sodium acetate buffer solution (pH 5.6) was incubated at room temperature. A further enzyme aliquot (3 ml) was added after achieving 30% lysis. 5-ml aliquots were sampled after lysis had reached 3, 13, 24, 42, 58, 84 and 100%, respectively; the reaction was monitored at 235 nm. Gel filtration was performed on a Biogel P-60 column (2 × 63 cm). The column was equilibrated and eluted with 0.2 M NaCl in 0.05 M phosphate buffer (pH 7.0). In each aliquot, the alginate content was estimated by the phenol/H_2SO_4 method [7] (approx. $\epsilon_{490} = 500$); absorbance at 235 nm was monitored ($\epsilon_{235} = 5000$) [8] to determine the number of end groups. (Fig. 1, Table I). The fractions were examined qualitatively by thin-layer chromatography on silica gel impregnated with 0.2 M Na_2HPO_4, eluted with ethanol/butanol/0.1 M H_3PO_4 (10 : 1 : 5, v/v) and developed by spraying with H_2SO_4.

Identification of uronic acid on reducing end of oligomers. Enzyme solution was added to 5 mg/ml alginate, 0.2 M NaCl 0.2% NaN_3 and 0.05 M acetate buffer (pH 5.6). After 3.5 days, more enzyme was added and the mixture was incubated for another two days. The mixture was subsequently chromatographed on a Biogel P-60 column (2.6 × 70 cm) in 0.2 M acetate buffer (pH 5.6). To ensure that complete lysis of the alginate had occurred, aliquots were taken, enzyme added and assayed at 235 nm (Table II).

$NaBH_4$ was added over 2 h at 25° C to a 25 mM oligomer solution (pH 9.0), polymerization degree 3—10. The borohydride was then degraded with acetic acid. The deionized and lyophilized preparation was hydrolyzed with 80% H_2SO_4 for 18 h at 20° C and then with 1 M H_2SO_4 for 5 h at 100° C [9]. After neutralization with $BaCO_3$, the mixture was chromatographed on Dowex 1 × 4 (acetate form 17—23 μm, 0.6 × 100 cm), eluted with 0.5 M acetic acid.

Determination of pH optimum. Enzyme activity was determined at pH 3—10 in an acetate/phosphate/borate (0.05 M each) buffer. The effects of ionic

strength (0.2 M NaCl) and Ca^{2+} (0.0075 M $CaCl_2$) were determined in 0.05 M Tris-acetate buffer using poly(ManUA) (1 mg/ml) as substrate, monitored at 235 nm (Fig. 2).

NMR spectra. To perform deuteration, 100 mg substrates were dissolved in 2.5 ml 0.2 M NaCl in 0.05 M acetate buffer (pH 5.6) and lyophilized; this was followed addition of 3 ml ^2H_2O, heating for 5 min at 60° C and lyophilizing three times. An ampoule was filled with 0.4 ml 40 mg/ml substrate solution and with approx. 15 μg lyophilized enzyme powder. The NMR spectra were recorded on an HX-90E Brucker instrument (F.R.G.). Ampoules were also filled with 0.5 ml 40 mg/ml substrate solution in ^2H_2O to record the spectrum at 80° C. After cooling the mixture to room temperature, 0.05 ml enzyme solution in ^2H_2O was added to the ampoule. The reaction was carried out at 30—35° C. Reaction product spectra were recorded at 80° C. Tertiary butanol was used as an internal standard, and chemical shifts were estimated in the σ scale with Me_4Si as external standard (Fig. 3).

Results and Discussion

On the basis of the relationship between viscosity and the reducing capacity of the incubation mixture, we assumed alginate lyase VI to be an endoalginate lyase [6]. A clearer criterion for determining the type of enzyme action has now been obtained by analysis of the reaction products. The initial reaction stages were characterized (Fig. 1, II—VIII) by increase of the high molecular weight peak; this was followed by appearance of fragments of medium-chain length. Accumulation of relatively short oligomers occurred in the last reaction stages. The low molecular weight peak constituted oligomers with polymerization degrees of 3—13 (Fig. 1, VIII, Table I). The polymerization degree was estimated in each fraction by the ratio of the number of sugars (phenol/H_2SO_4 method) to the number of end groups (A_{235}). The fragments of low molecular weight appeared only after 60—100% hydrolysis. These data were confirmed by the results of thin-layer chromatography on silica gel, showing mobile spots of unidentified oligomers below the monouronic acids used as standards, only after 60—100% lysis.

The above pattern (absence of short oligomers in the initial reaction stages and gradual decrease in the molecular weight of the polymer substrate) is typical of an enzyme with an endo-type action.

Highly-polymerised alginic acid is known to contain fragments with polymerization degrees up to 20, containing solely D-mannuronic or L-guluronic acids residues (poly(ManUA) or poly(GulUA) respectively) and also sites with alternating sequences of both monomers (poly(ManUA-GulUA)) [14]. Apparently, the presence of fragments of high and medium molecular weight in the lysis products serves as an indication of the high specificity of the enzyme.

It should be noted that, in most cases, polyuronide lyases have an optimum pH in the range 7—8 [1—5], and alginate lyase VI shows maximum activity at pH 5.6 in 0.05 M buffer. A more detailed study showed the enzyme pH-dependency pattern to be complex (Fig. 2).

Increase in ionic strength and introduction into the mixture of 0.0075 M Ca^{2+} resulted in an increase in alginate lyase VI activity, with a maximum at pH

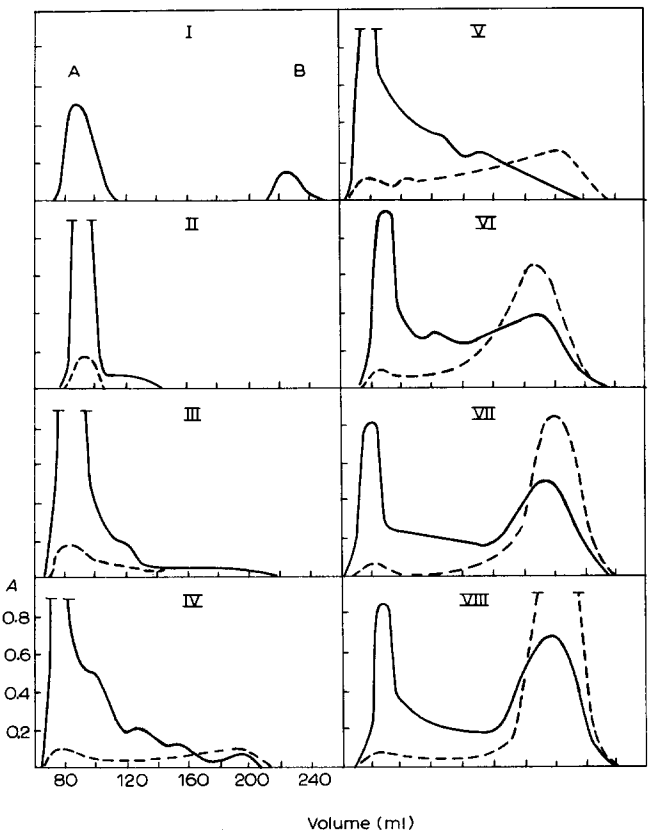

Fig. 1. Gel filtration asssay of alginic acid enzymolysis products under action of alginate lyase VI. After performing enzymolysis to various depths (II, 3%, III, 13%; IV, 24%; V, 42%; VI, 58%; VII, 84%; VIII, 100%), the reaction products were chromatographed on Biogel P-60 (2.6 × 70 cm). The fractions were analyzed by phenol H_2SO_4 method (———) and photometered at 235 nm (- - - - -). I, column calibration (A, Dextran blue 2000; B, galacturonic acid).

TABLE I

DETERMINATION OF POLYMERIZATION DEGREE OF ALGINIC ACID OLIGOMERS AFTER LYSIS

Alginic acid concentration (sugar concentration) was determined by calibration curve.

Fractions	Sugar concentration (mM)	Concentration of end groups (mM)	Degree of polymerization
170	0.44	0.033	13.3
175	0.61	0.062	9.8
180	0.81	0.086	9.5
190	1.36	0.18	7.6
200	1.29	0.21	6.1
210	0.73	0.15	4.9
220	0.20	0.053	3.7

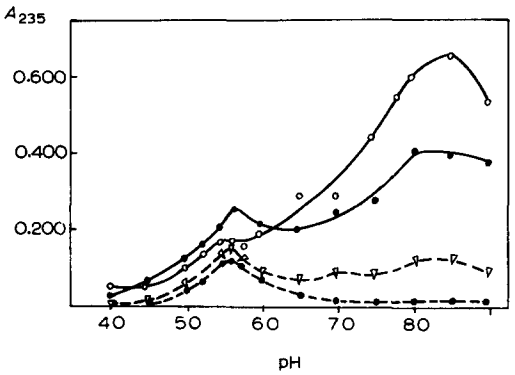

Fig. 2. Effect of ions on pH dependency of alginate lyase VI. Activity was determined using poly(ManUA) as substrates and by photometering at 235 nm. •------•, 0.05 M acetate/phosphate/borate buffer; ▽------▽, 0.05 M Tris-acetate buffer; •———•, 0.2 M NaCl in 0.05 M Tris-acetate buffer; ○———○, 0.0075 M $CaCl_2$ in 0.05 M Tris-acetate buffer.

8.0—8.5. The pattern of dependency of the enzyme activity of alginate lyase VI upon pH, ionic strength and the presence of Ca^{2+} was found to be very similar to that in one of the pectin lyases [10]. Cations decreased the surface density of the substrate charge to weaken the ionic interaction between the alginate (polyanion) and the enzyme (polycation), with isoelectric point at pH 8.15 [6].

Some of the data previously obtained, including the K_m and V values, for hydrolysis of substrates with different composition, poly(GulUA) resistance and presence of large oligomers in the products of exhaustive lysis of the polymer substrate, suggests that alginate lyase VI not only cleaves the bonds between two mannuronic acid residues, but also actively attacks poly(ManUA-GulUA) and the alginate, both of which contain all four types of linkage. The enzyme's specificity was, therefore, still in doubt.

A similar situation was observed when we examined alginase preparations isolated from *Dolabella* [1] and *Pseudomonas* sp. [2]. Enzyme specificity was chiefly assessed by poly(GulUA) or poly(ManUA) resistance to the action of the polymannuronide lyase (or, correspondingly, to that of the polyguluronide lyase), or by the relative rates of cleavage of substrates of varying compositions [11]. The same difficulties were observed in establishing heparinase specificity in respect to D-glucuronic and L-iduronic acids [3,4].

A detailed study of the polyguluronide lyase from *Pseudomonas* sp. revealed that, following hydrolysis, complete acid hydrolysis, neutralization and chromatography, the ManUA/GulUA ratio changed from 1.9 in the initial substrate to 0.1 in the exhaustively lysed products. It seems surprising that a considerable decrease was observed in the monouronic acid yields, from 30% in the initial substrate to 1% after a 3-day incubation with the enzyme [5].

To establish the type of cleaved bond, we determined the residue on the reducing end in the products. Completion of the reaction was determined by an absence of any increase in A_{235} after adding a fresh enzyme aliquot not only to the reaction mixture, but also to a solution of oligomers isolated by gel filtration from a separate incubation (Table II). Short oligomers with polymeriza-

TABLE II

DETERMINATION OF COMPLETENESS OF ENZYMOLYSIS

Increase in absorbance was determined after adding enzyme in fraction following gel filtration of exhaustive enzymolysis products. Fractions as in Fig. 1, VIII. The A_{235} values are cited after definite intervals of incubation time.

Fractions (ml)	A_{235}		
	0 h	3 h	12 h
70	0.110	0.110	0.105
90	0.100	0.100	0.100
140	0.098	0.093	0.100
170	0.095	0.095	0.098
190	0.080	0.085	0.085
220	0.005	0.005	0.005

tion degrees of 3—10 were reduced with $NaBH_4$ and then subjected to exhaustive acid hydrolysis [9]. The resultant mixture was subjected to anion-exchange chromatography on a column with Dowex 1X4. Among the aldonic acids that form as a result of such treatment, only mannonic acid was detected on the reducing end of the oligomers. It was identified by chromatographic behaviour during ion-exchange chromatography and thin-layer chromatography, compared to an authentic sample, and also by the absence of reducing capacity. As in the control test, gluconic acid was present in trace amounts. Its formation was associated with epimerization of mannuronic acid during the course of reduction and hydrolysis.

The residue on the non-reducing end of the resultant oligomers was determined by NMR. On examining the initial substrates poly(ManUA), poly(GulUA) or poly(ManUA-GulUA), spectra similar to those described earlier were obtained (Fig. 3) [12]. In the poly(ManUA) spectrum at low field, an intense signal was observed at σ = 4.87 ppm, τ = 2—3 Hz; no other signal in the range of 4.5—6.0 ppm, was observed, indicating homogeneity of the preparation. In the poly(GulUA) spectrum, two signals with σ = 5.22 and 4.63 ppm were observed; these are assigned to the anomeric proton at C-1 and to the proton at C-5. The poly(ManUA-GulUA) spectrum was found to have signals due to mannuronic and guluronic acids.

In the spectra of the poly(ManUA) and poly(ManUA-GulUA) lysis products, a double-bond signal (σ = 5.98 ppm, τ = 4 Hz) occurred, indicative of a lyase action mechanism in alginate lyase VI; an unidentified signal with σ = 5.40 ppm was also observed. Furthermore, enzymic cleavage of poly(ManUA-GulUA) was found to result in noticeable variations (from 1 : 5 to 1 : 1.2) in the intensity ratios of the proton signals with σ = 5.22 and 4.83 ppm of the guluronic and mannuronic acids, respectively. In this case, no marked decrease was noted in the intensity of the peak with σ = 5.22 ppm (guluronic acid). These results indicate that, in poly(ManUA-GulUA) the enzyme caused elimination only of mannuronic acid residues; hence, alginate lyase VI did not affect the ManUA-GulUA glycoside bonds.

It was only quite recently that ^{13}C-NMR was used for alginate studies. The

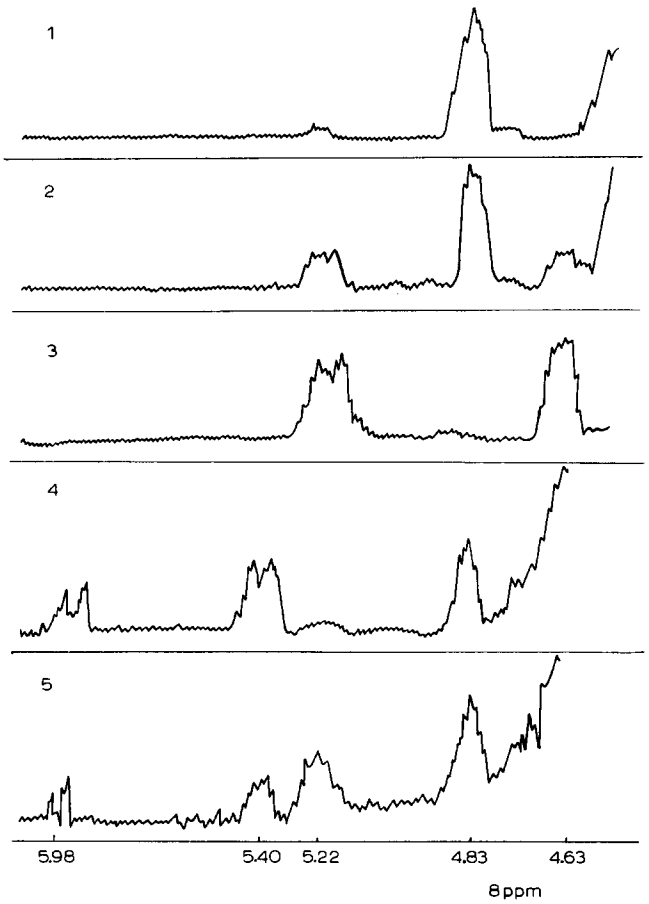

Fig. 3. NMR spectra of substrates and alginate lyase VI end products. 1, Poly(ManUA); 2, poly(ManUA-GulUA); 3, poly(GulUA); 4, products of poly(ManUA) splitting by alginate lyase VI; 5, products of poly-(ManUA-GulUA) splitting by alginate lyase VI.

method can distinguish signals from trimers with different composition [13], and hence affords the possibility of unambiguously determining the specificity of alginate lyases.

Thus, from the fact that poly(GulUA) is not subject to the action of alginate lyase VI, one may conclude that the enzyme does not split the GulUA-GulUA glycoside bond. Absence of guluronic acid on the reducing end of the resultant oligomers indicates that the GulUA-ManUA linkage is not affected. NMR data show that alginate lyase VI does not split the ManUA-GulUA bond. Hence, the enzyme alginate lyase VI is essentially a polymannuronide lyase with an endo-type action and splits solely ManUA-ManUA bonds.

References

1 Nakada, H. and Sweeny, P. (1967) J. Biol. Chem. 242, 845—851
2 Fujibayashi, S., Habe, H. and Nisizawa, K. (1970) J. Biochem. (Tokyo) 67, 37—45
3 Linker, A. and Hoving, F. (1970) Biochemistry 11, 563—572

4 Suzuki, S., Saito, N., Yamagata, T., Anno, K., Seno, N., Kawai, Y. and Furuhashi, T. (1968) J. Biol. Chem. 243, 1543—1550
5 Min, K.H., Sasaki, S.F., Kashiwabara, Y., Umekawa, M. and Nisizawa, K. (1977) J. Biochem. (Tokyo) 81, 555—562
6 Elyakova, L.A. and Favorov, V.V. (1974) Biochim. Biophys. Acta 398, 341—354
7 Dubois, M., Gilles, K.A., Hamilton, J.R., Roberts, P. and Smith, F. (1956) Anal. Chem. 28, 350—356
8 Tsujino, J. (1963) Agric. Biol. Chem. 27, 236—242
9 Haug, A. and Larsen, B. (1962) Acta Chem. Scand. 16, 1908—1918
10 Edstrom, R.D. and Phaff, H.J. (1964) J. Biol. Chem. 239, 2409—2415
11 Davidson, I.W., Sutherland, I.W. and Lawson, C.J. (1976) Biochem. J. 159, 707—713
12 Pennan, A. and Sanderson, G.R. (1972) Carbohydr. Res. 25, 273—282
13 Grasdalen, H., Larsen, B. and Smidsrod, O. (1977) Carbohydr. Res. 56, C11—C15
14 Haug, A., Larsen, B. and Smidsrod, O. (1966) Acta Chem. Scand. 20, 183—190